Attachment A
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ENRICHMENT METHOD FOR GROWTH HORMONE VARIANTS WITH ALTERED BINDING PROPERTIES

This is a continuation-in-part of U.S. serial No. 07/621,667, filed 3 December 1990.

FIELD OF THE INVENTION

This invention relates to the preparation and systematic selection of novel binding proteins having altered binding properties for a target molecule. Specifically, this invention relates to methods for producing foreign polypeptides mimicking the binding activity of naturally occurring binding partners. In preferred embodiments, the invention is directed to the preparation of therapeutic or diagnostic compounds that mimic proteins or nonpeptidyl molecules such a hormones, drugs and other small molecules, particularly biologically active molecules, such as growth hormone.

BACKGROUND OF THE INVENTION

Binding partners are substances that specifically bind to one another, usually through noncovalent interactions. Examples of binding partners include ligand-receptor, antibody-antigen, drug-target, and enzyme-substrate interactions. Binding partners are extremely useful in both therapeutic and diagnostic fields.

Binding partners have been produced in the past by a variety of methods including; harvesting them from nature (e.g., antibody-antigen, and ligand-receptor pairings) and by adventitious identification (e.g. traditional

which they are rendered resistant due to the presence of tet and/or amp resistance genes on the vector.

After selection of the transformed cells, these cells are grown in culture and the plasmid DNA (or other vector with the foreign gene inserted) is then isolated. Plasmid DNA can be isolated using methods known in the art. Two suitable methods are the small scale preparation of DNA and the large-scale preparation of DNA as described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. The isolated DNA can be purified by methods known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*. This purified plasmid DNA is then analyzed by restriction mapping and/or DNA sequencing. DNA sequencing is generally performed by either the method of Messing *et al.* (Nucleic Acids Res., 9:309 [1981]) or by the method of Maxam *et al.* (Meth. Enzymol., 65: 499 [1980]).

IV. Gene Fusion

This invention contemplates fusing the gene enclosing the desired polypeptide (gene 1) to a second gene (gene 2) such that a fusion protein is generated during transcription. Gene 2 is typically a coat protein gene of a phage, and preferably it is the phage M13 gene III coat protein, or a fragment thereof. Fusion of genes 1 and 2 may be accomplished by inserting gene 2 into a particular site on a plasmid that contains gene 1, or by inserting gene 1 into a particular site on a plasmid that contains gene 2.

Insertion of a gene into a plasmid requires that the plasmid be cut at the precise location that the gene is to be inserted. Thus, there must be a restriction endonuclease site at this location (preferably a unique site such that the plasmid will only be cut at a single location during restriction endonuclease digestion). The plasmid is digested, phosphatased, and purified as described above. The gene is then inserted into this linearized plasmid by ligating the two DNAs together. Ligation can be accomplished if the ends of the plasmid are compatible with the ends of the gene to be inserted. If the restriction enzymes are used to cut the plasmid and isolate the gene to be inserted create blunt ends or compatible sticky ends, the DNAs can be ligated together directly using a ligase such as bacteriophage T4 DNA ligase and incubating the mixture at 16°C for 1-4 hours in the presence of ATP and ligase buffer as described in section 1.68 of Sambrook *et al.*, supra. If the ends are not compatible, they must first be made blunt by using the Klenow fragment of DNA polymerase I or bacteriophage T4 DNA polymerase, both of which

require the four deoxyribonucleotide triphosphates to fill-in overhanging single-stranded ends of the digested DNA. Alternatively, the ends may be blunted using a nuclease such as nuclease S1 or mung-bean nuclease, both of which function by cutting back the overhanging single strands of DNA. The DNA is then religated using a ligase as described above. In some cases, it may not be possible to blunt the ends of the gene to be inserted, as the reading frame of the coding region will be altered. To overcome this problem, oligonucleotide linkers may be used. The linkers serve as a bridge to connect the plasmid to the gene to be inserted. These linkers can be made synthetically as double stranded or single stranded DNA using standard methods. The linkers have one end that is compatible with the ends of the gene to be inserted; the linkers are first ligated to this gene using ligation methods described above. The other end of the linkers is designed to be compatible with the plasmid for ligation. In designing the linkers, care must be taken to not destroy the reading frame of the gene to be inserted or the reading frame of the gene contained on the plasmid. In some cases, it may be necessary to design the linkers such that they code for part of an amino acid, or such that they code for one or more amino acids.

Between gene 1 and gene2, DNA encoding a termination codon may be inserted, such termination codons are UAG(amber), UAA (ocher) and UGA (opel). (Microbiology, Davis et al. Harper & Row, New York, 1980, pages 237, 245-47 and 274). The termination codon expressed in a wild type host cell results in the synthesis of the gene 1 protein product without the gene 2 protein attached. However, growth in a suppressor host cell results in the synthesis of detectible quantities of fused protein. Such suppressor host cells contain a tRNA modified to insert an amino acid in the termination codon position of the mRNA thereby resulting in production of detectible amounts of the fusion protein. Such suppressor host cells are well known and described, such as E.coli suppressor strain (Bullock et al., BioTechniques 5, 376-379 [1987])). Any acceptable method may be used to place such a termination codon into the mRNA encoding the fusion polyupeptide.

The suppressible codon may be inserted between the first gene encoding a polypeptide, and a second gene encoding at least a portion of a phage coat protein. Alternatively, the suppressible termination codon may be inserted adjacent to the fusion site by replacing the last amino acid triplet in the polypeptide or the first amino acid in the phage coat protein. When the